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Determination of gp120 & Trx80 dependent production of hydrogen peroxide in cell free & cell-dependent systems

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ABSTRACT

Hydrogen peroxide (H_2O_2), a reactive oxygen specie (ROS), is most commonly associated with oxidative stress causing cytotoxic effects on living cells. Oxidative stress has been implicated in various conditions including neurodegenerative diseases, autoimmune diseases and cancer. In addition H_2O_2 is produced as a defense mechanism against pathogens, as being released by activated phagocytes. In recent years, H_2O_2 has become established as an important regulator of signal transduction in eukaryotic cells. Hydrogen peroxide is generated both intracellularly and extracellularly in response to various stimuli including cytokines and growth factors. There are different mechanisms by which H_2O_2 is generated, facilitating signal transduction in cells; through NOX-system in miyochondria, via singlet oxygen, receptor/ligand interaction or by redox active metal ions. The HIV glycoprotein 120 (gp120) is associated with HIV dementia and it is known as a neurotoxin that causes neuronal damage. It has been proposed that free radicals may be involved in the pathogenesis caused by gp120. In addition the truncated form of thioredoxin (Trx80) is known to stimulate HIV replication in HIV infected cells, however, the exact mechanism is not known. A possible way both proteins may mediate their activity is by inducing H_2O_2 production. The aim of this study was to investigate H_2O_2 production induced by the proteins gp120 and Trx80. In order to detect H_2O_2 production an assay based on the fluorescent compound Amplex Red, was established. The assay was used to detect H_2O_2 released by gp120 and Trx80 in a cell-free environment, in a cell-system and in the presence of metal ions (copper ions) with a physiological reductant (ascorbate). We did not detect H_2O_2 production induced by gp120 and Trx80 respectively, using our assay, however, other ROS such as hydroxyl radicals may have been generated although they were not detectable with our method. Hence, further studies are needed in order to fully understand how gp120 and Trx80 mediate their activity.

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ABBREVIATIONS

O_2	Molecular oxygen
$^1O_2$	Singlet oxygen
$O_2^{\bullet -}$	Superoxide
H_2O_2	Hydrogen peroxide
Cu	Copper
CD	Cluster of differentiation
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGF/R	Epidermal growth factor/receptor
FBS	Fetal bovine serum
gp120	HIV glycoprotein 120 subunit
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IL	Interleukin
NOX	NADPH oxidase
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Trx	Thioredoxin

1. INTRODUCTION

1.1 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is one of the known reactive oxygen species (ROS) together with other reactive molecules such as superoxide radical, hydroxyl radical and nitric oxide. H_2O_2 is commonly associated with oxidative stress, when the normal redox state of a cell is disturbed. High concentrations of free radicals from H_2O_2 and other ROS is damaging to cells, causing oxidative modification of biomolecules including nucleic acids, proteins, carbohydrates and lipids [1]. This oxidative damage is thought to be involved in several inflammatory diseases, carcinogenesis, atherosclerosis and aging [2]. H_2O_2 is commonly generated in the mitochondrial matrix with the help of mitochondrial superoxidase dismutase, but can also be released by phagocytes as a defence mechanism against pathogens [3]. However, studies from recent decades indicate new roles of hydrogen peroxide in biological systems, as being an important regulator of signal transduction in nonphagocytic cells [4]. Hydrogen peroxide may be generated both extracellularly by proteins such as antibodies, in receptor/ligand interactions [5, 6], through redox active metal ions and intracellularly through superoxide dismutation in mitochondria (Figure 1).

Hydrogen peroxide is generated in several different ways. It is deliberately produced by phagocytes when experiencing oxidative or respiratory burst (Figure 1.1) [3]. This is characterized by a large increase of oxygen consumption and generation of ROS, upon recognition of stimulus when encountering inflammatory mediators and pathogens. The production of ROS, including H_2O_2, is generated through the activated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX). Electrons generated by NOX

mediate the reduction of molecular oxygen to form superoxide ($O_2^{\bullet-}$), which subsequently is converted either spontaneously or by superoxide dismutase (SOD) into H_2O_2 [3].

$$NADPH + O_2 \rightarrow NADP^+ + H^+ + O_2^{\bullet-}$$

$$O_2^{\bullet-} \rightarrow \text{dismutates spontaneously or by SOD} \rightarrow H_2O_2$$

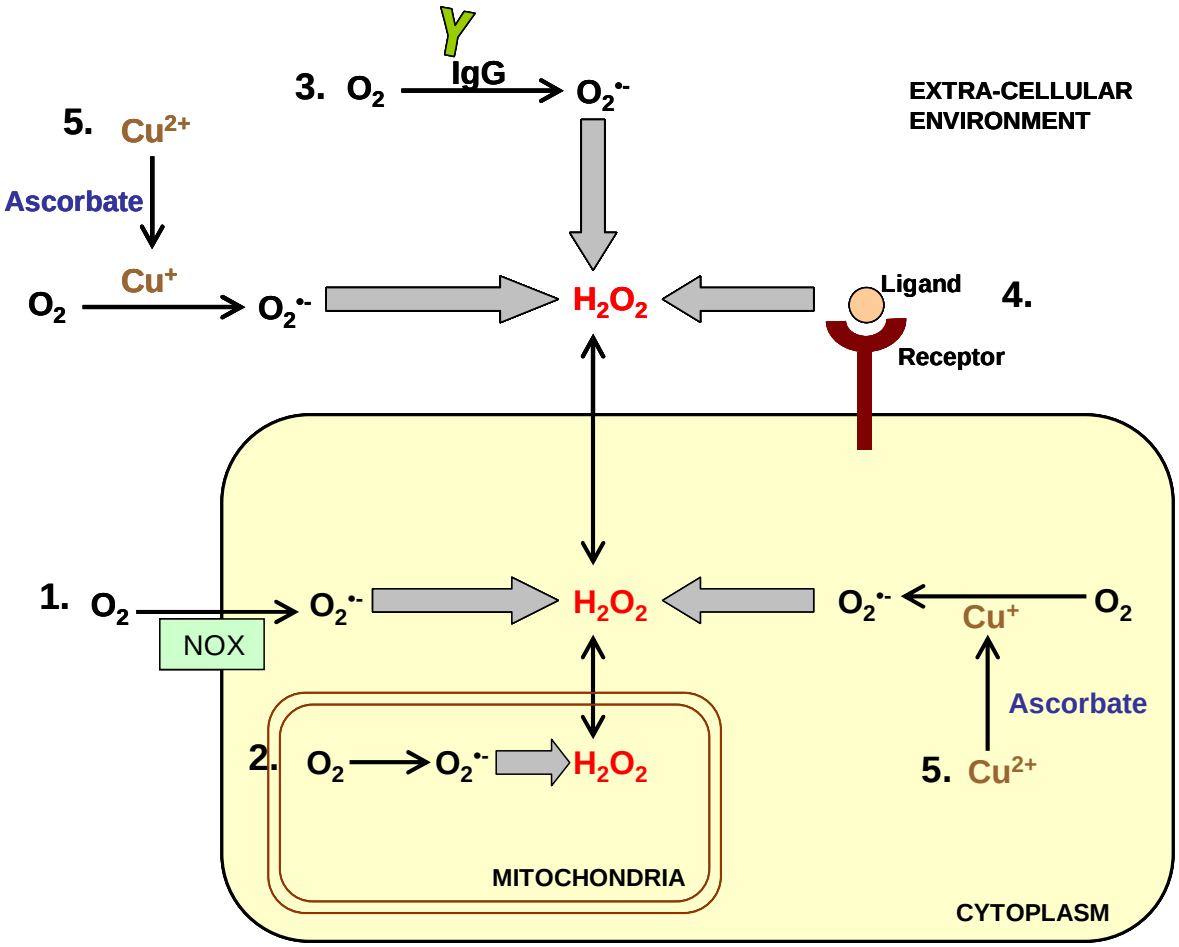


Figure 1 Schematic illustration of different mechanisms of H_2O_2 production in cells. H_2O_2 is produced: 1. through NOX in phagocytes, 2. in mitochondria, 3. through antibodies, 4. via receptor/ligand interaction and 5. through metal ion reduction.

Hydrogen peroxide is also continuously generated under normal physiological conditions, being an inevitable by-product of the aerobic respiration process in cells (Figure 1.2). Almost 95% of cellular oxygen is reduced to water through electron carriers in the mitochondria

respiratory chain, however when adenosine diphosphate (ADP) concentration is low a small amount of oxygen may leak out forming superoxide, which subsequently is converted into H_2O_2 [7, 8].

Recent studies show that hydrogen peroxide can be produced by antibodies (Figure 1.3). Richard Lerner *et al.* have shown that antibodies have the intrinsic ability to reduce singlet oxygen ($^1O_2$) into superoxide ($O_2^{\bullet-}$) which is converted into H_2O_2 [9]. This previously unknown chemical defence mechanism of the immune system is intrinsic to antibody molecules, allowing oxygen to be recycled from activated phagocytes and potentiating the oxygen-dependent microbicidal action.

$$^1O_2 \rightarrow \text{IgG} \rightarrow O_2^{\bullet-} \rightarrow \text{dismutases spontaneously into } H_2O_2$$

A third way by which H_2O_2 can be produced is through receptor/ligand interactions (Figure 1.4). One example is the epidermal growth factor (EGF) and its receptor (EGFR), which upon ligand binding, generates H_2O_2 extracellularly [6].

Another mechanism by which H_2O_2 can be produced is by metal ion chemistry (Figure 1.5), via the Fenton's reaction. Redox active metal ions, in particular copper and irons ions do react with physiological reductants such as ascorbate, reducing dicationic ions (metal ion gaining one electron). The reduced metal ions can then react with molecular oxygen yielding superoxide, subsequently yielding various ROS including H_2O_2.

$$\text{Free } Cu^{2+} + \text{Ascorbate} \rightarrow Cu^{+} + \text{dehydroxyascorbate (oxidized ascorbate)}$$

$$Cu^{+} + O_2 \rightarrow O_2^{\bullet-} \text{ (formation of superoxide radicals)}$$

$$O_2^{\bullet-} + O_2^{\bullet-} \rightarrow H_2O_2 \text{ (formation of hydrogen peroxide)}$$

Proteins such as amyloid plaque, prion proteins and amylin [11-15] have been shown to generate radicals through this mechanism (described further below).

1.2 Hydrogen peroxide as a signalling agent

Being a mild oxidant, H_2O_2 also plays a role as an intracellular signalling agent, affecting the function of various proteins, transcription factors, enzymes, ion channels and G-proteins [10]. By oxidizing proteins with low-pK_a (dissociation constant) cysteine-residues, H_2O_2 can activate or inactivate proteins both in cells such as protein tyrosine phosphatases (PTPs) which are involved in cell signalling pathways [10] and NF-κB activation [11].

Hydrogen peroxide also functions as an extracellular signalling agent through monocytes oxidative burst where intracellular generated H_2O_2 is released into the extracellular environment which affects other cells [3]. In addition H_2O_2 generated extracellularly through receptor/ligand interactions by for instance the EGF receptor [6] can cross the cell membrane and thereby facilitate receptor-mediated signal transduction in the cell [6] (Figure 2).

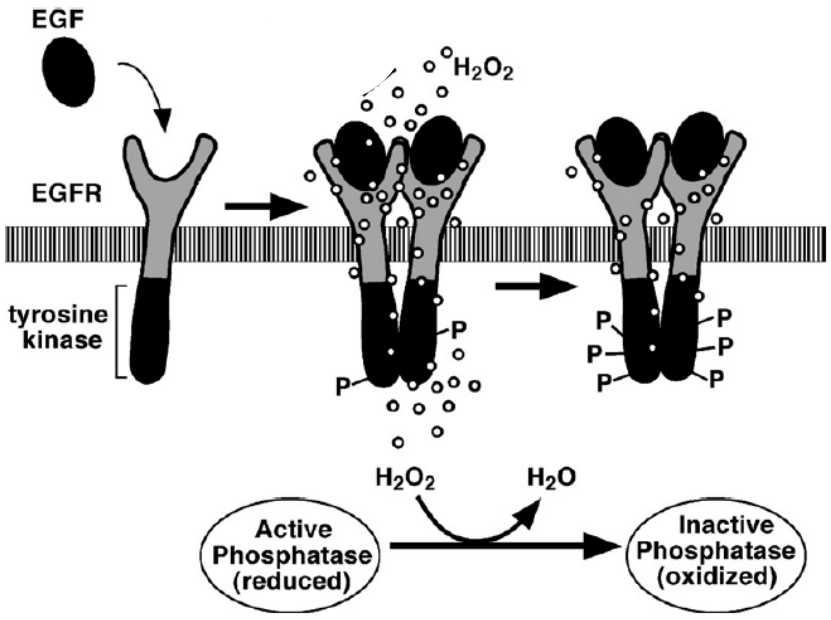


Figure 2 Proposed model of extracellular H_2O_2 mediated signal transduction generated by EGF-EGFR interaction. The H_2O_2 that is generated upon ligand binding modulates EGFR-dependent signal transduction cascades by inactivating EGFR-related phosphatases. (Adapted from G.J. De Yulia Jr, J.M. Cárcamo, 2005)

Several different proteins, including cytokines and growth factors initiate signalling pathways by binding to receptors, ROS such as H_2O_2 have been shown to modulate these signalling cascades. However the exact mechanism through which H_2O_2 is generated leading to signal transduction is not known.

A possible mechanism by which proteins can generate H_2O_2 extracellularly is via singlet oxygen. Singlet oxygen can in turn be reduced to superoxide by for example antibodies and thereby generate H_2O_2, after being activated by a photoactivator such as ultraviolet light. This pathway has been shown by Richard Lerner *et al.* regarding antibodies and αβ T-cell receptor (αβTCR) [9, 12].

Another way H_2O_2 can be generated by proteins, both intra- and extracellularly, is via redox active metal ions mentioned earlier. Proteins such as amyloid plaque [13, 14] , prion proteins [15, 16] and amylin peptide [17] have been shown to generate H_2O_2 by reducing dicationic copper ions. Copper ions have a plasma concentration of approximately 0.1-1μM and around 250nM in the cerebrospinal fluid [18]. Reduced metal ions can react with molecular oxygen (O_2), forming a superoxide radical, which can generate H_2O_2, the generated H_2O_2 can together with metal ions drive the Fenton's reaction and thereby generate hydroxyl radicals.

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{\bullet -} \text{ (generation of superoxide radicals)}$$

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H \rightarrow H_2O_2 + O_2 \text{ (production of hydrogen peroxide)}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^{-} \text{ (Fenton's chemistry, hydroxyl radical formation)}$$

1.3 HIV glycoprotein 120

The structural envelope glycoprotein 120 subunit (gp120) of Human Immunodeficiency Virus-1 (HIV) is one of the viral proteins that have been identified as a neurotoxin causing

neuronal cell death in HIV infected patients, leading to HIV associated dementia [19, 20]. The HIV enters host cells via the binding of gp120 to CD4 [21]. Although the virus does not infect neuronal cells, they are affected indirectly by infected microglia and macrophages in the brain [22-24]. Upon infection, viral proteins such as gp120, gp41 and Tat are released into the extracellular environment [25]. There are several mechanisms by which these proteins become available in the extracellular environment; when the viral coat is shed upon viral entry and when the infected cell is ruptured releasing virus particles, the unincorporated structural viral proteins may be transported out of the infected cells and proteins can also be actively secreted from the infected cells [26] (Figure 3).

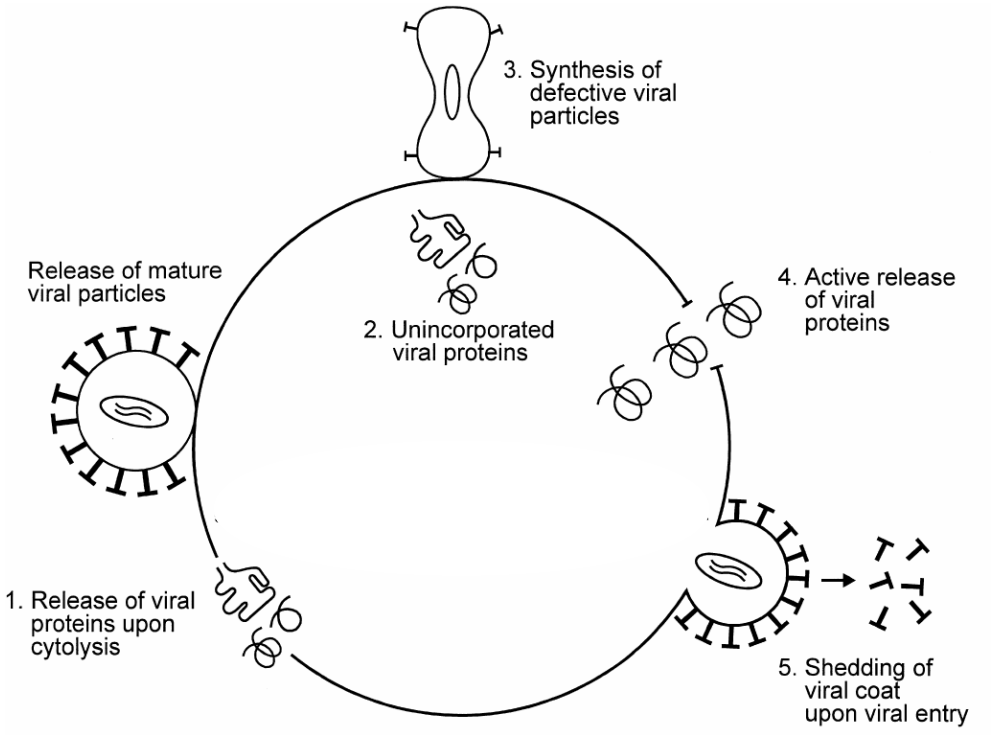


Figure 3 Potential mechanisms by which viral proteins (such as gp120) may become available to the extracellular environment from HIV infected cells. (Adapted from Avindra N. *et. al*, 1997)

Gp120 has been shown to cause apoptotic cell death at only picomolar concentrations in rat ganglion cells, rat neurons, astrocytes, human neurons, etc [27-30]. The neurotoxic effects of gp120 is indirect and is mediated by affecting astrocytes and microglia [31]. It also induces

the production of various cytokines such as tumour necrosis factor TNFα and interleukin 1 (IL-1) [32]. These chemokines and cytokines further activate uninfected microglia and macrophages, causing them to release neurotoxins, which eventually lead to neuronal damage and apoptotic cell death [33, 34]. Gp120 may also cause cytotoxicity by inducing oxidative stress, studies show that gp120 is able to produce free radicals leading to oxidative damage on monocytoid cells [35]. However the exact mechanism behind the hydrogen peroxide generated by gp120 is still not known.

1.4 Truncated Thioredoxin

Thioredoxin-1 (Trx1) is a small soluble 12-kDa protein which has a characteristic dithiol active site motif; Cys-Gly-Pro-Cys. The motif is evolutionary conserved and is found in many different plants and organisms [36]. Trx1 was first discovered in *Escherichia coli* as a hydrogen donor for the reductive enzyme ribonucleotide reductase by Peter Reichard *et al.* [37]. It has later been established that Trx1 has several different substrates including ribonuclease. Further more it also acts as disulfide reductase for fibrinogen, insulin and chorigonaotropin etc [38-40]. Trx1 is involved in many biochemical processes in the cell, it plays an important role as a redox regulator together with glutaredoxins (Grxs), reducing protein disulfides and can thereby maintain a reduced environment inside of cells even under severe oxidative stress. Trx1 is also involved in reducing hydrogen peroxide, through thioredoxin peroxidase, thus preventing oxidative stress and also inhibiting the induction of apoptosis [41, 42].

A truncated form of Trx1 was first identified as the eosinophilic enhancing factor (ECEF), which was found when studying eosinophil stimulating activity in the supernatant of human blood mononuclear cell (PBMC) culture of patients suffering from schistosomiasis [43, 44].

Two proteins 10 kDa and 12 kDa respectively were isolated and found to be identical to Trx [45]. In 1989 Silberstein *et al.* [46] found that the 10 kDa protein had the strongest eosinophilic cytotoxicity enhancing activity, and it was established that the ECEF activity was induced from a C-terminally truncated form of the full length Trx consisting of either the 80 (Trx80) or 84 (Trx84) N-terminal amino acids of Trx [47].

Trx80 is mostly secreted by monocytes although T-lymphocytes, activated platelets, cytotrophoblasts and EBV+ B-cells also produce Trx80 [46, 48-50]. The generation of Trx80 *in vivo* is not yet fully known, however it is believed that Trx is cleaved on the cell surface by an unknown enzyme [51], the truncated form subsequently binds to the membrane of macrophages [52]. In solution Trx80 is present as a homodimer [53].

Except for sharing the same 80 N-terminal amino acid sequence with Trx1, Trx80 has almost no functional similarity to it. Trx80 does not catalyze the reduction of insulin disulfides by dithiothreitol (DTT) which is a characteristic activity of Thioredoxins [47, 53, 54], in addition it is not a substrate for thioredoxin reductase and it also seems to lack redox activity [53].

Being a mitogenic cytokine for PBMC in culture, Trx80 has shown to act as a cytokine since it by itself stimulates proliferation of PBMC, in contrast to Trx1 which has no cell proliferating effect on human PBMC [53]. Trx80 enhances CD14 expression of monocytes [55]. CD14 is a surface receptor important for the removal of apoptotic cells etc., hence Trx80 induced up-regulation of CD14 may stimulate phagocytosis of apoptotic cells and microbes [55]. Expression of several other surface antigens is also increased when monocytes are treated with Trx80, such as CD40, CD54 and CD86 [55]. Trx80 also induces the expression of CD40 on monocytes, this up-regulation of CD40 may stimulate IL-12 and IFN-γ production inducing a T-helper response in T-lymphocytes. The up-regulation of CD40, IL-12

and IFN- γ could promote development of autoimmune diseases such as multiple sclerosis and arthritis [56-58]. In addition Trx80 has been reported to have stimulating effect on HIV replication in infected macrophages [52], in contrast to Trx1 which inhibits viral replication in HIV infected cells [44]. Nevertheless the exact mechanism of Trx80 stimulating activity on HIV replication is still not known.

1.5 Aims of the study

Besides from being involved in oxidative stress, hydrogen peroxide has shown to play a role as a signalling agent in cells [4]. H_2O_2 can be generated as a result of receptor/ligand interaction extracellularly [6], by antibodies through photoactivation [9] and via metal ion reduction (through Fenton's reaction) [13, 14, 17]. Today the mechanisms by which gp120 exerts its cytotoxicity on neuronal cells are not fully understood, but available data support the fact that gp120 induces the production of free radicals such as H_2O_2 [35], suggesting that oxidative stress may be involved. In addition it is not known how Trx80 mediates its activity by which it induces HIV replication in HIV infected macrophages [52]. A possible way both proteins may mediate their activity is by the generation of H_2O_2, hence the overall aim of this study was to investigate H_2O_2 production induced by gp120 and Trx80. In order to detect H_2O_2 production an assay based on the commercial kit Amplex Red Hydrogen Peroxide was established for *in vivo* and *in vitro* detection of hydrogen peroxide.

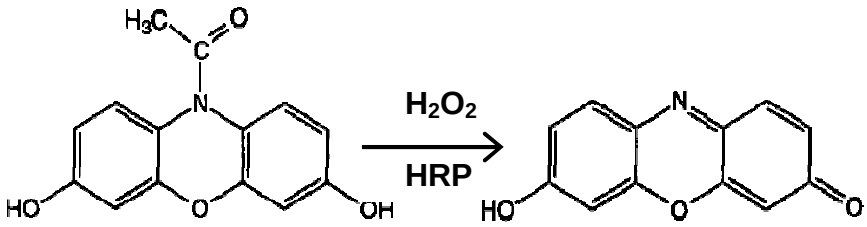
2. MATERIALS *&* METHODS

2.1 Measuring H_2O_2

In general ROS are highly reactive and have short life time [59] which makes them difficult to measure directly in biological systems. Hence more indirect methods are needed to assess levels of ROS. One method is to use molecular probes that are oxidatively modified, which allows detection with luminescence or fluorescence. However the methods that detect free radicals have low sensitivity and specificity due to their short life time and the possibility of antioxidants to capture the ROS etc. [59]. A recent method that has shown to be more stable and sensitive than other assays [60], is based on the non-fluorescent compound *N*-acetyl-3,7-dihydroxyphenoxazine, referred as Amplex Red [60, 61]. This assay was used in the present study to measure H_2O_2.

Amplex Red Hydrogen Peroxide Assay

The Amplex Red Hydrogen Peroxide assay (Molecular Probes, A22188) is based on the non-fluorescent compound *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red). In the presence of horse radish peroxidise (HRP), Amplex Red is oxidized by H_2O_2 with 1:1 stoichiometry yielding the highly red fluorescent compound resorufin [61] (Figure. 3). This compound has an excitation maximum at 563 nm and emission maximum at 587 nm, allowing detection of hydrogen peroxide fluorometrically and spectrophotometrically at very low concentrations (from 5pM).



Amplex Red
(*N*-Acetyl-3,7-Dihydroxyphenoxazine)

Non-fluorescent

Resorufin
(7-Hydroxy-3H-Phenoxazine-3-one)

Highly fluorescent

λ_{ex} = 563 nm
λ_{em} = 587 nm

Figure 4 Horseradish peroxidase (HRP) catalyzed H_2O_2 oxidation of Amplex Red into the highly fluorescent compund resorufin, detectable at excitation wavelength 563 nm and emission at 587 nm.

Stock solutions of Amplex Red (10 mM) in dimethyl sulfoxide (DMSO) and HRP (10 units/mL) in phosphate buffered saline (PBS) were stored at -20°C. Fresh working solutions of Amplex Red (100µM) and HRP (0.2 units/mL) were prepared for each experiment. The fluorescence readings were measured with InfiniteM1000 plate reader (TECAN), with excitation at 544nm and emission at 590nm. Standard curve of known H_2O_2 concentrations was used to quantitate the produced H_2O_2.

2.2 Determination of gp120 & Trx80 induced H_2O_2 production

Based on the fact that proteins are able to produce H_2O_2 (such as the EGF receptor [6]), both in a cell-free environment and in cell-systems, we aimed to measure the release of H_2O_2 by the proteins gp120 and Trx80. The proteins gp120 (Immuno Diagnostics Inc.) and Trx80 (IMCO Corp.) at 0.5µM concentration diluted in PBS in a 50µL reaction were incubated for 60 minutes at 37C, prior to measurement, 50µL fresh working solution of Amplex Red was added to the samples and the fluorescence was measured.

In addition the ability of gp120 and Trx80 to induce H_2O_2 production was also investigated through Fenton's chemistry. Proteins such as amyloid plaque, prion proteins and amylin have shown to be able to induce H_2O_2 production [13, 14, 17]. Hence we aimed to investigate if gp120 and Trx80 are able to induce H_2O_2 through this mechanism. Fresh stock solutions were prepared for each experiment, each 50μL reaction mixture in PBS contained 0.25μM $CuCl_2$ (Sigma), 30μM ascorbate (Sigma) and one of the proteins gp120 and Trx80 in different concentrations. As a control EDTA (50μM) was added. EDTA is a chelating agent that can bind different di- and tricationic metal ions, thus free Cu^{2+} present in the samples would be chelated and no H_2O_2 would be produced. Samples were incubated for one hour at 37°C, after the incubation ascorbate was added and the samples were further incubated for 15 minutes. Then 50μL fresh working solution of Amplex Red was added to each reaction mixture and fluorescence readings were measured.

2.4 Cell experiments

For the present study immortalised cell lines were used in order to study the effects of gp120 and Trx80 *in vivo*. Although primary cell lines closest resembles physiological environment and morphology, they are subject to large variation between individual donors, in addition they require addition of specific growth factors and can only be passaged limited times. Thus immortalized cell lines, which are easier to grow and propagate, can yield reproducible data and are more cost effective, were used in this study.

Selection of cell lines

T-cells are the major target for HIV due to its high abundance of the cell-surface receptor CD4. There are several cell-lines such as JURKAT, MOLT and CEM cells which can be used to study T-cell responses. Given that there are only small differences between these cell lines,

The CEM cell line was used in this study. This cell line expresses the antigens CD3, CD4, CD5 and CD7 which are typical for T-cells. Other cell-types that can also be infected by HIV include monocytes, macrophages and astrocytes, we therefore studied the effects on monocytes, using MonoMac 6 (MM6) cells. MM6 unlike some other monocytic cell lines expresses the antigen CD14 which binds endotoxins, a hallmark for monocytes, making these cells more suitable for this study. Other cell lines that can be used to study monocytes are U937 and THP1 cells, however these cells lack the expression of some cell surface receptors and are supposed to be less similar to primary monocytes.

Cell culture

CEM cells (ATCC) were maintained in RPMI 1640 media (Gibco Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco),100 units/mL penicillin (Sigma) and 0.1mg/mL streptomycin (Gibco) at cell densities 2-3x10^5 cells/mL. The MM6 cells (kindly provided by Prof. Olof Rådmark, Karolinska Institute) were maintained in RPMI 1640 media supplemented with 10% FBS, 1x non essential aminoacids (Gibco), 1mM sodium pyruvate (Gibco), 10ug/mL bovine insulin (Sigma), 100 units/mL penicillin and 100µg/mL streptomycin at cell densities 0.3-1.0x10^6 cells/mL. The cells were cultured at 37°C, 5% CO_2.

2.5 Release of H_2O_2 by gp120 & Trx80 stimulated cells

Studies show that cells treated with proteins (such as EGF and gp120), are able to induce H_2O_2 production [6, 35]. Hence we aimed to test if gp120 and Trx80 are able to induce H_2O_2 production in T-cells (using CEM-cells) and monocytes (using MM6-cells). Prior to the experiments cells were harvested by centrifugation (700*g*, 5 min), washed twice and resuspended in phospate buffered saline (PBS) to appropriate concentrations. A concentration of 2x10^5 cells/mL in a reaction mixture of total 50µL, were incubated in black flat-bottomed

96-well plates (Nunc) with or without the proteins; gp120 (250pM) and Trx80 (250pM) in PBS at 37°C for four hours.

As a control catalase (1 000 units/mL, Sigma) was added, catalase is an enzyme that catalyzes hydrogen peroxide into water and oxygen, thus the fluorescent signal should be reduced. In order to measure the released H_2O_2, 50µL working solution of Amplex Red were added to the samples, the fluorescence was measured at 30
minutes intervals.

3. RESULTS

3.1 Hydrogen peroxide assay

In order to study the production of H_2O_2 induced by the proteins gp120 and Trx80, an assay based on the fluorescent probe Amplex was set up. A standard curve was set up with known concentrations of H_2O_2 (Figure 5A). In addition as a control test, catalase was added to known amount of H_2O_2, as expected the fluorescent signal was reduced with the addition of catalase (Figure 5B). The stability of the assay was tested by measuring a known amount of H_2O_2 over time (Figure 5C), the results show a decrease of fluorescent signal over time.

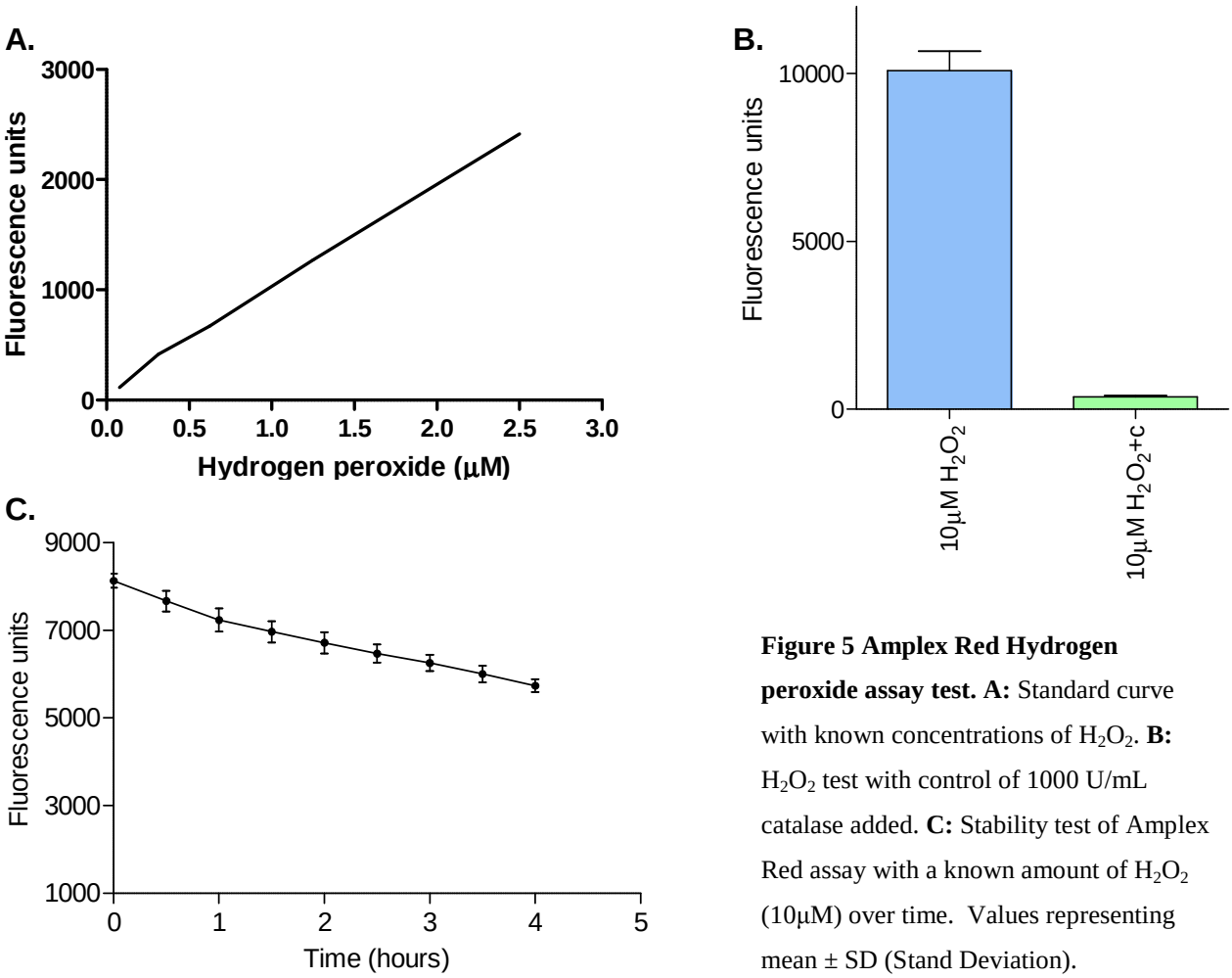


Figure 5 Amplex Red Hydrogen peroxide assay test. A: Standard curve with known concentrations of H_2O_2. **B:** H_2O_2 test with control of 1000 U/mL catalase added. **C:** Stability test of Amplex Red assay with a known amount of H_2O_2 (10µM) over time. Values representing mean ± SD (Stand Deviation).

3.2 Determination of gp120 & Trx80 induced H_2O_2 production

The ability of gp120 and Trx80 to induce H_2O_2 in a cell-free environment was also investigated in this study. The proteins were incubated for 60 minutes at 37°C and the amount of produced H_2O_2 was measured using Amplex Red (Figure 6). The results show that the proteins did not produce H_2O_2, since the samples gave signals on the same level as the control or below.

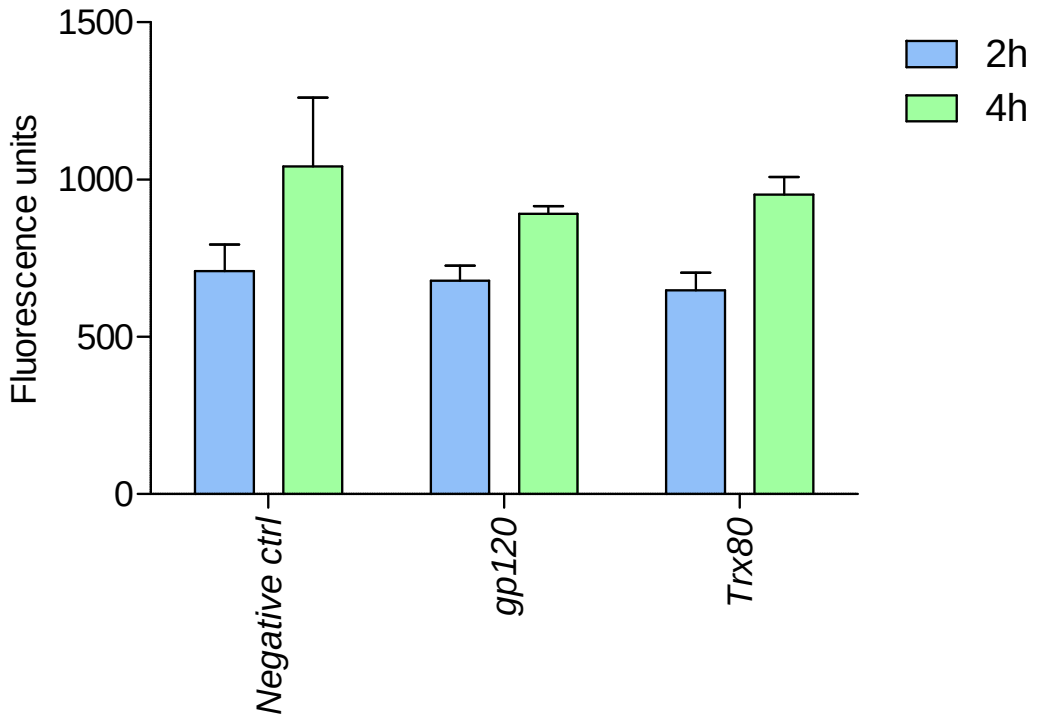


Figure 6 H_2O_2 production of gp120 and Trx80 in cell-free environment using Amplex Red assay. The proteins (0.5µM) in PBS were incubated for 60 minutes at 37°C, prior to reagent addition. The negative control consist of PBS only. Values representing mean ± SD.

To test if gp120 and Trx80 were able to mediate activity by inducing H_2O_2 production in T-cells and monocytes, CEM-cells and MM6-cells a concentration of $2x10^5$/mL with or without the proteins gp120 and Trx80 (250pM) and the control samples, were incubated for four hours

at 37°C and the amount of H_2O_2 was measured continuously. The results of the test are represented in Figure 7 and 8, where the amount of H_2O_2 released after two and four hours are shown. The results showed no difference in H_2O_2 production after four hours incubation and no difference between the two cell lines. Gp120 and Trx80 with or without cells produced a small amount of H_2O_2. However cells treated with proteins did not produce more H2O2 than the control, indicating no or very little H_2O_2 production. As an additional control, catalase (1 000 units/mL) was added to the samples, and in the positive sample (1μM H_2O_2) the signal was decreased with the addition of catalase, confirming that it is the H_2O_2 that caused the positive signal.

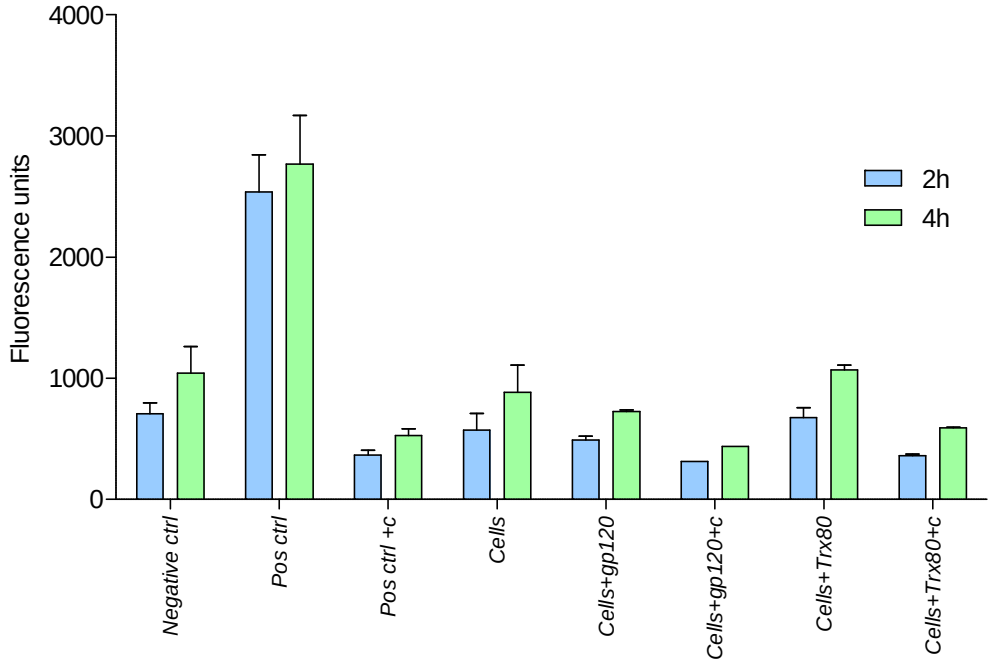


Figure 7 H_2O_2 production in CEM cells treated with gp120 and Trx80 using Amplex Red assay. Control samples and samples with cells at concentration $2x10^5$ cells/mL were incubated with or without the proteins gp120 or Trx80 (250pM) and catalase (1 000 units/mL) for four hours at 37°C. (Negative ctrl: Negative control (PBS), Pos ctrl: positive control (1µM H_2O_2), c= catalase. Values representing mean ± SD.

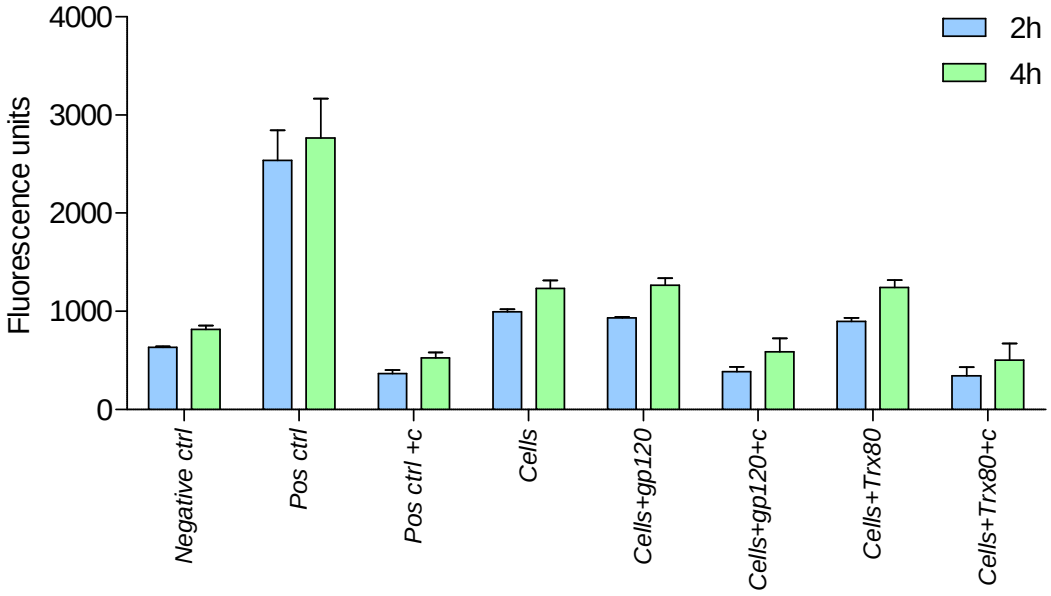


Figure 8 H_2O_2 production in MM6 cells treated with gp120 and Trx80 using Amplex Red assay. Control samples and samples with cells at concentration $2x10^5$ cells/mL were incubated with or without the proteins gp120 or Trx80 (250pM) and catalase (1 000 units/mL) for four hours at 37°C. Negative ctrl: Negative control (PBS), Pos ctrl: positive control (1µM H_2O_2), c= catalase. Values representing mean ± SD.

A third way we examined the capacity of gp120 and Trx80 to induce H_2O_2 production was by incubating them with metal ions and a reductant such as ascorbate. Previous studies with proteins such as amyloid plaque and prion proteins have been shown to be able to induce H_2O_2 production by reducing redox active metals such as copper and iron ions [13, 14, 17]. Hence we aimed to measure if the proteins gp120 and Trx80 have the ability to reduce dicationic copper and through this mechanism induce hydrogen peroxide production. After incubating the proteins (0.5μM) with copper (0.25μM) for 60 minutes, ascorbate (30μM) was added to the samples and incubated for 15 minutes prior to measurement with Amplex Red working solution. Figure 9 shows the results of selected controls and protein samples. As expected, the control samples such as copper or buffer alone did not generate any hydrogen peroxide. Ascorbate gave a slight decrease in fluorescence which may be due to interactions with assay reagents. The control sample with copper in the presence of ascorbate yielded high amounts of H_2O_2 (3.0μM), as expected. In order to confirm that H_2O_2 is produced due to metal ion reduction with ascorbate, EDTA was added, which decreased the signal down to baseline levels as expected. The proteins gp120 and Trx80 in the absence of copper ions and ascorbate gave no increase in H_2O_2 production. However the proteins together with ascorbate gave no signal either, indicating no production of H_2O_2 with proteins alone or in the presence of the reductant ascorbate at physiological levels. With the addition of both copper ions and ascorbate, both gp120 and Trx80 gave low signals, suggesting that the proteins had bound the copper ions that were present, hence no free copper ions were left for ascorbate to generate H_2O_2. However Trx80 yielded slightly higher amounts of H_2O_2 (1.5μM) compared to gp120.

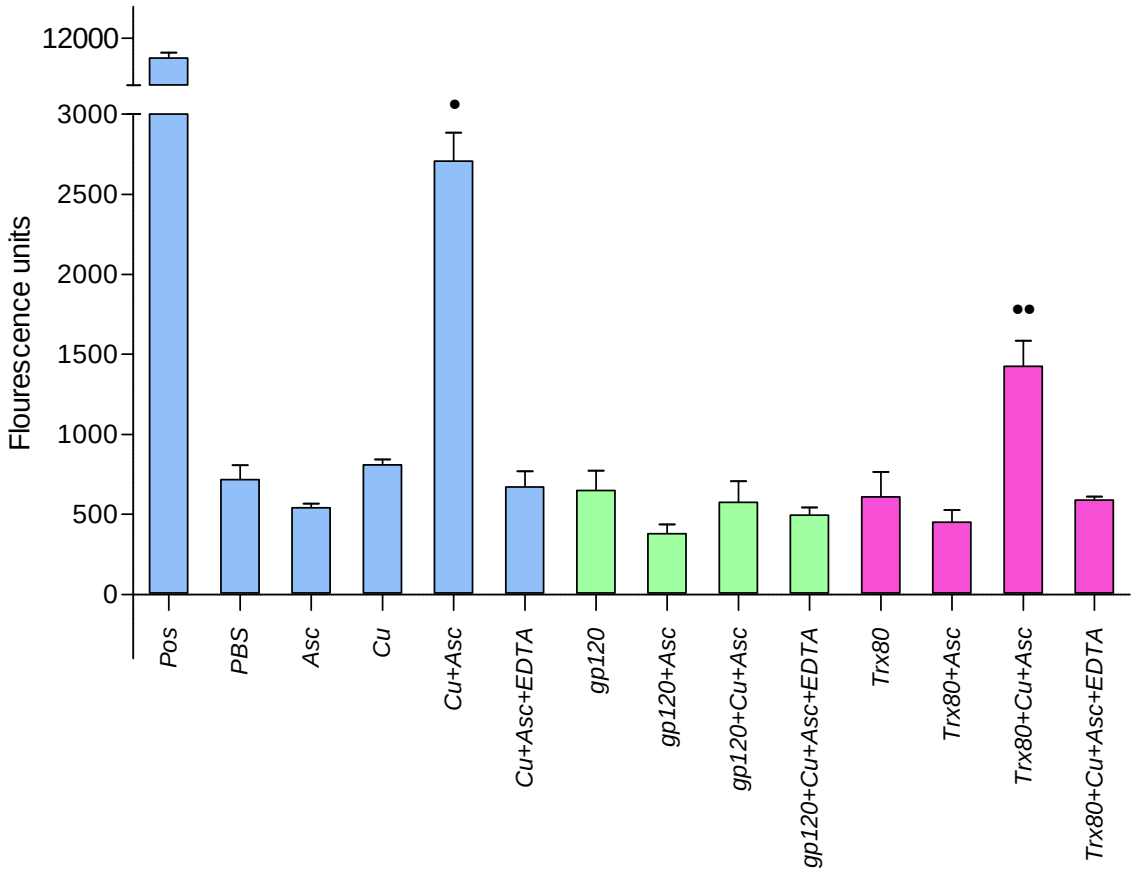


Figure 9 H_2O_2 production by Cu^{2+} and ascorbate in the presence of gp120 and Trx80, monitored using Amplex Red assay. Gp120/Trx80 (0.5µM) with or without $CuCl_2$ (0.25µM) and ascorbate (30µM) in various combinations, and control samples; positive control (5µM H_2O_2), and samples with EDTA (50µM) were incubated for 60 minutes at 37°C. After further incubation with ascorbate for 15 minutes, Amplex Red working solution was added and fluorescence was measured. ●= 3.0µM H_2O_2 yielded by copper ions and ascorbate, ●●= 1.5µM H_2O_2 yielded by Trx80 in the presence of copper ions and ascorbate. (PBS= Phosphate buffered saline, pos= positive control (10µM H_2O_2), Asc: ascorbate, Cu: $CuCl_2$). Values representing mean ± SD.

In order to determine to what extent the proteins are able to bind copper ions, a dilution test was performed with decreasing concentrations of gp120 and Trx80. Gp120 at different concentrations from 0.5μM to 312.5nM were incubated for 60 minutes with 0.25 μM $CuCl_2$, at 37°C. Subsequently the samples were incubated with the addition of ascorbate for 15 minutes, whereupon the amount of H_2O_2 was detected with Amplex Red working solution (see Figure 10). As a result of high amounts of gp120, no free copper ions are left to react with ascorbate thus no H_2O_2 was produced. However, decreasing concentrations of gp120 led to an increased signal of H_2O_2 production, further strengthening that with less amount of the metal ion binding protein, more copper is free and consequently more H_2O_2 is produced in the presence of ascorbate. These data suggest that gp120 is not able to induce the formation of H_2O_2, since copper ions that bind to the protein are then not free to induce H_2O_2 production in the presence of ascorbate.

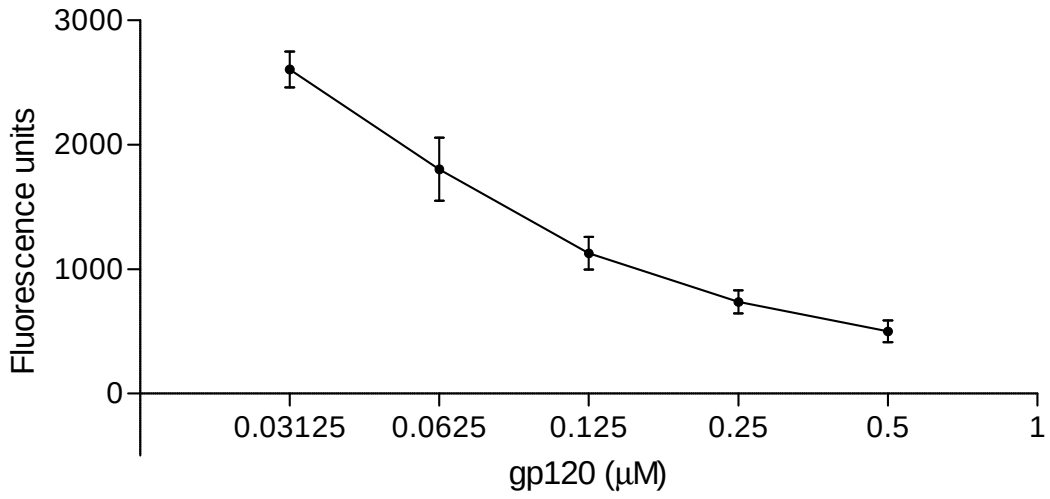


Figure 10 Dilution test with protein gp120, H_2O_2 production in the presence of Cu^{2+} and ascorbate using Amplex Red assay. Gp120 at various concentrations (0.5μM-312.5nM) were incubated with 0.25μM $CuCl_2$ at 37°C for 60 minutes and additional 15 minutes with 30μM ascorbate, prior to reagent addition.

A similar test was performed with Trx80, where decreasing concentrations of Trx80 were incubated with copper and ascorbate (Figure 11). In contrast to gp120, Trx80 do not seem to have similar linearity of increasing H_2O_2 production with increasing amount of Trx80. A scavenging activity was only observed for Trx80 at the highest concentration tested 0.5μM.

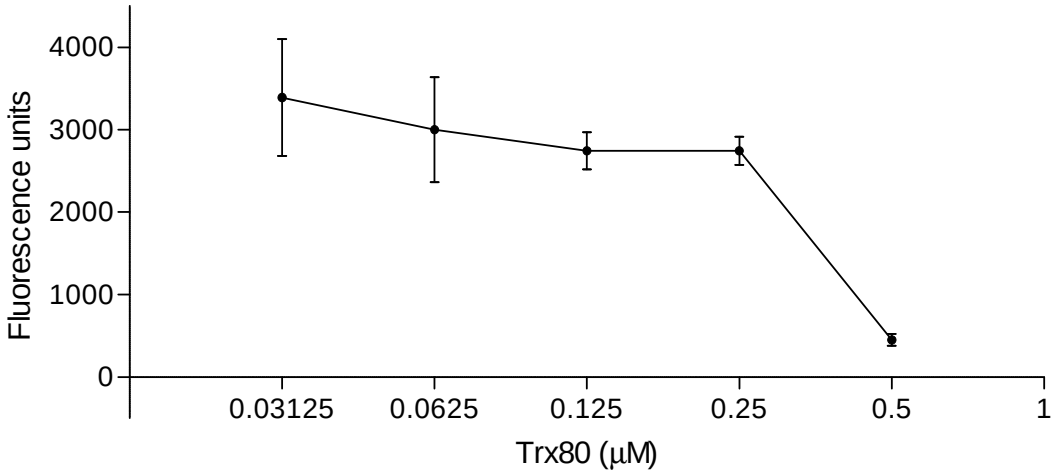


Figure 11 Dilution test with protein Trx80, H_2O_2 production in the presence of Cu^{2+} and ascorbate using Amplex Red assay. Trx80 at various concentrations (312.5nM-0.5μM) were incubated with 0.25μM $CuCl_2$ at 37°C for 60 minutes and an additional 15 minutes with 30μM ascorbate, prior to reagent addition.

4. DISCUSSION

The generation of reactive oxidative species such as hydrogen peroxide has been implicated in various diseases from neurodegenerative diseases and cancer to autoimmune diseases such as diabetes, primarily by inducing oxidative stress. Previous data support that hydrogen peroxide can also be implicated as a signalling agent, facilitating signal transduction in cells [4]. Based on the previous studies supporting free radical production of gp120 [35], the aim of this study was to investigate the mechanism of H_2O_2 production induced by this protein. In addition we aimed to investigate whether Trx80 mediate its biological activity by inducing H_2O_2 as previously observed for a number of growth factors [5]. In order to test these hypotheses we established a commercial available assay to determine H_2O_2 production. As mentioned earlier, numerous assays have been developed for the determination of H_2O_2. We decided to develop an assay based on the Amplex Red reagent, a reagent that shifts fluorescence upon binding to H_2O_2. The assay was shown to be easy to use, sensitive and stable. In the present work we have not tested the specificity of the assay, whether it is only reacting with H_2O_2 and not with other ROS. However, previous work by others have shown that the assay in its present setup is specific [61]. Although the compound Amplex Red used in this study is highly stable (see Figure 6), with higher concentrations of H_2O_2 there is a risk that the fluorescent signal from resorufin (reduced Amplex Red) is lost due to a second oxidation step, converting resorufin into a non-fluorescent compound such as resazurin (see Figure 12) [60]. However this was avoided by maintaining the molar ratio between Amplex Red and H_2O_2 above five in our reaction mixtures, ensuring linear dose response of hydrogen peroxide. In addition the variations in fluorescent readings between individual experiments can be explained by differences in the stock solutions of Amplex Red. Amplex Red reagent is highly light

sensitive and can spontaneously oxidize resulting in differences in background fluorescence, hence fresh working solutions of Amplex Red were prepared for each experiment.

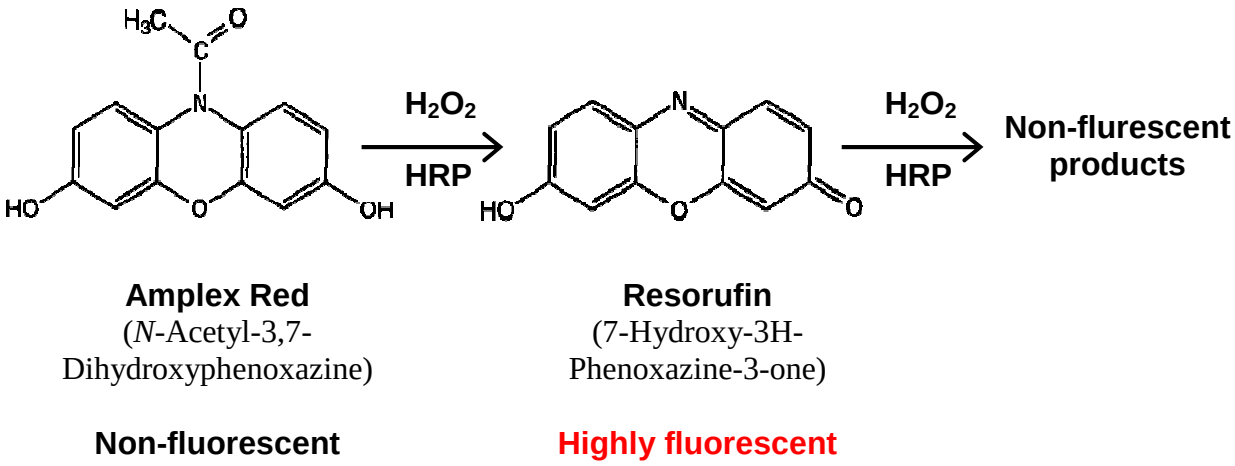


Figure 12 Reaction scheme for the oxidation of Amplex Red catalyzed by horseradish peroxidase (HRP) and H_2O_2.

With Amplex Red assay we subsequently tested whether gp120 and Trx80 were able to produce H_2O_2. In a cell-free environment we were not able to detect any H_2O_2 induced by either gp120 or Trx80. Previous reports have shown that antibodies, T-cell receptor αβ (αβTCR) and some cytokines growth factors have the ability to produce H_2O_2 in cell free systems [12]. However, for antibodies and αβTCR the H_2O_2 production was shown to be dependent on reaction power from singlet oxygen to the proteins. This was obtained by artificial generation of singlet oxygen, by exposing dissolved molecular oxygen in the protein solution to ultraviolet light or photoactivators [9, 12]. H_2O_2 has been shown to be produced by cytokines and growth factors alone [5], but the H_2O_2 production can be further induced when the cytokines and growth factors are bound to their specific receptors. In addition we were not able to see any H_2O_2 after binding gp120 with its biological receptor CD4 (data not shown). In the present report we did not test gp120 and Trx80 dependent H_2O_2 in the occurrence of singlet oxygen. The previous studies were also conducted with Amplex Red reagent to determine H_2O_2 which limits the possibility that the results are due to methodological reasons. However, further studies needs to be performed to clarify this issue.

Previous studies have shown that numerous metal binding proteins, for instance amyloid protein and prions, may induce H_2O_2 in mixtures with oxygen and antioxidants such as glutathione, L-cysteine and ascorbate [13]. We tested whether gp120 and Trx80 were able to produce H_2O_2 in mixtures with oxygen and copper or iron. However, we were not able to observe any H_2O_2 production related to these proteins. Unexpectedly, we observed strong and dose-dependent reduction of H_2O_2 production in mixtures with gp120. This result suggests that gp120 binds copper, which has not been described before. Moreover, bound copper appears to be unable to react to oxygen and antioxidants. This result may suggest that gp120 is unable to produce H_2O_2 in environments with free copper and antioxidants (i.e. extra cellular environment). The relevance of copper bound to gp120 needs to be further investigated.

The ability of gp120 and Trx80 to induce H_2O_2 production in T-cells, which may actively secrete H_2O_2, was also tested in this study. Our results indicated no H_2O_2 production after exposure to gp120 and Trx80, respectively, by the tested cells. For gp120 this result was rather unexpected since H_2O_2 production has been reported after gp120 exposure [35]. In that study, the observed H_2O_2 was shown to be strongly reduced by EDTA and catalase. The effect of EDTA may indicate that the production was dependent on the occurrence of metals. This result may be due to contaminating metals in the assay. In the reported study by Foga *et al.* [35], another assay was used to detect hydrogen peroxide, the thiobarbituric acid-reactive species (TBARS) was used to measure oxidative damage of gp120 on cells. This assay has been shown to be less specific also notified by the authors of the report. Their result may be due to detection of other ROS species than hydrogen peroxide. In this regard it would be interesting to analyse other ROS species (i.e. hydroxyl radicals) after exposure of gp120 and Trx80. As described in the introduction of the present report, hydrogen peroxide is rapidly converted to hydroxyl radicals in the presence of metals by Fenton reaction. There is thus a

possibility that the hydrogen peroxide produced may have already been converted into hydroxyl radicals. Hence it can not be concluded that no free radicals were produced, since we have only detected H_2O_2.

With the present study we can conclude that gp120 or Trx80 do not seem to have the ability to induce H_2O_2 production, as we tested the proteins in our Amplex Red assay in both cell-free environment, in a cell system and in the presence of metal ions. Hence, to fully understand all the mechanisms by which gp120 exerts its cytotoxicity in HIV associated dementia and how Trx80 mediates its activity by which it induces HIV replication in cells, further studies are needed in this field.

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